

**CHARACTERIZATION OF *Salvia bogotensis* ANTI-LECTIN IGYS
AND THEIR APPLICATION IN IMMUNOCYTOCHEMICAL STUDIES
INVOLVING TN ANTIGEN DETECTION**

**CARACTERIZACIÓN DE IGYS ANTI-LECTINA DE *Salvia bogotensis*
Y SU APLICACIÓN EN ESTUDIOS CITOQUÍMICOS PARA LA DETECCIÓN
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E A SUA APLICAÇÃO EM ESTUDOS CITOQUÍMICOS PARA A DETECÇÃO
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Nohora Vega¹, Hansen Murcia¹, Gerardo Pérez^{1, 2}

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ABSTRACT

Immunoglobulins isolated from egg yolk (IgY) are tools which are currently used in different fields of the biological sciences; they have clear advantages over mammalian serum IgGs. We have established the conditions for obtaining anti-*Salvia bogotensis* lectin IgYs in previous work; their use in immunocytochemical studies requires that their main molecular characteristics are known as well as the conditions for IgY-lectin interaction. *Salvia bogotensis* lectin (SBoL) can specifically recognise Tn antigen, a recognised tumoral marker in many types of cancer but additional tools are required for evidencing this interaction in cells. Given the availability of *S. bogotensis*

anti-lectin IgY, this work was aimed at molecularly characterising these IgYs and evaluating their application in immunocytochemical studies for detecting Tn antigen in tumour cells. Purified IgYs' isoelectric points, molecular weight and carbohydrate content were determined. Homologous and heterologous lectins were obtained for establishing the specificity of antibody interaction with the lectin; they were assayed by ELLSA. Biotin or peroxidase-labelled IgYs were prepared; Tn antigen was specifically detected by CELISA and immunocytochemistry in MCF-7 and HeLa cell-lines with the lectin which was revealed with the labelled IgYs. Results showed that anti-SBoL IgY antibodies represent a highly sensitive

¹ Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia, sede Bogotá, Bogotá, Colombia.

² jrperezg@unal.edu.co

tool for specific Tn antigen recognition assays.

Key words: Lamiaceae, *Salvia bogotensis*, lectin, IgY, Tn antigen, immunocytochemistry.

RESUMEN

Las inmunoglobulinas aisladas de la yema de huevo (IgY) son muy utilizadas actualmente en diversos campos de las ciencias biológicas, dadas sus ventajas frente a las IgG séricas de mamíferos. En un trabajo previo establecimos las condiciones de obtención de IgY dirigidas contra la lectina de *Salvia bogotensis*; su utilización en estudios inmunocitoquímicos requiere conocer sus principales características moleculares y las condiciones para la interacción IgY-lectina. La lectina de *Salvia bogotensis* (SBoL) reconoce específicamente el antígeno Tn, marcador tumoral en muchos tipos de cáncer, pero se requieren herramientas adicionales para evidenciar esta interacción a nivel celular. Dada la disponibilidad de IgY anti-lectina de *S. bogotensis*, se realizó este trabajo con el objeto de caracterizar molecularmente estas IgY y evaluar su utilización en estudios inmunocitoquímicos para la detección del antígeno Tn en células tumorales. A las IgY purificadas se les determinó su punto isoeléctrico, peso molecular y contenido de carbohidratos. Para establecer la especificidad de interacción IgY-SBoL se obtuvieron lectinas homólogas y heterólogas y se ensayaron por ELLSA. La detección del antígeno Tn en las líneas celulares MCF-7 y HeLa con la lectina y las IgYs marcadas con biotina o peroxidasa se realizó por CELISA e inmunocitoquímica. Los resultados mostraron que los anticuerpos

IgY anti-SBoL son una herramienta de una alta sensibilidad para los ensayos de reconocimiento específico del antígeno Tn.

Palabras clave: Lamiaceae, *Salvia bogotensis*, lectina, IgY, antígeno Tn, inmunocitoquímica.

RESUMO

Imunoglobulinas isoladas a partir de gema de ovo (IgY) são ferramentas utilizadas atualmente em diferentes áreas das ciências biológicas y apresentam vantagens claras sobre o soro de mamífero IgGs. Em investigações anteriores estabelecemos as condições para obter lectina IgYs anti-*Salvia bogotensis*. O seu uso em estudos imunocitoquímicos requer que as suas principais características moleculares sejam conhecidas, assim como as condições para a interação IgY-lectina. A lectina de *Salvia bogotensis* (SBoL) pode reconhecer especificamente o antígeno Tn, um reconhecido marcador tumoral em muitos tipos de cancro, mas novas ferramentas são necessárias para evidenciar esta interação em células. Dada a disponibilidade de anti-lectina IgY de *S. bogotensis*, este trabalho teve como objectivo a caracterização molecular de estes IgYs e avaliar a sua aplicação em estudos imunocitoquímicos para detectar antígeno Tn em células tumorais. Foram determinados os pontos isoeléctricos, peso molecular e conteúdo de carbohidratos de IgYs purificadas. Lectinas homologas e heterologas foram obtidas para estabelecer a especificidade da interação de anticorpo com a lectina; estes foram ensaiados por ELLSA. Foram preparados IgYs marcados com biotina ou peroxidase. O antígeno Tn foi detectado

especificamente por CELISA e imunocitoquímica em linhas celulares MCF-7 y HeLa com lectina que foi revelada com as IgYs marcadas. Os resultados mostraram que os anticorpos anti-SBoL IgY representam uma ferramenta altamente específica para ensaios de reconhecimento específico de antígeno Tn.

Palavras-chave: Lamiaceae, *Salvia bogotensis*, lectina, IgY, antígeno Tn, imunocitoquímica.

INTRODUCTION

Just like mammals, birds produce antibodies in response to an antigen and have been used as an experimental model since the beginnings of basic research in immunology; mammals and birds' immune systems have many functional similarities (1, 2).

Chickens have been the most widely used experimental model; as well as a high IgM type molecular weight protein and a IgA type being found in chickens' secretions, blood plasma and oviduct (3, 4), antibodies analogous to IgG called IgY have also been found. This term was proposed by Leslie and Clem (5) for denoting some particular characteristics differentiating IgG from mammals from birds' IgY. The latter are transferred to the egg-yolk via the ovarian follicular epithelium during oogenesis during a process which is similar to placental transference as happens in mammals (6). This leads to greater IgY concentrations in the yolk than in serum; values of up to 25-45 mg of immunoglobulins per ml of yolk (7, 8) can be obtained, having recuperation of up to 10 mg of antibody/ml yolk (8), depending on the isolation method. Given that there is no evi-

ce about the presence of IgM or IgA in yolk, IgYs are the only antibodies present in it whilst IgM and IgA have been found in very small amounts in egg-white and in the amniotic liquid and digestive tract of bird embryos (6).

The most notable molecular difference between IgG and IgY lies in their molecular weight which is in the order of 180 kDa for the latter. Each IgY heavy chain (δ chain) has a 67-70 kDa molecular weight and each light chain weighs 25 kDa. Their greater size compared to the γ chain of the IgGs (50 kDa) or the α chain of the IgAs (60 kDa) is due to the presence (besides the variable domain (V)) of four constant domains (CH1, CH2, CH3 and CH4) by contrast with IgGs' γ chain which only consists of 3 constant domains (9).

Immunoglobulins can coexist in their complete form and in a truncated form (120 kDa, 5.7s) within the same individual, as happens in some groups of turtles (*Pseudamys spp.*) (10) and some anseriforms or may only present one type of form, as in chickens which produce the complete form and some groups of amphibians, reptiles and turtles exclusively producing the truncated form (11). They also lack a hinge region (11) meaning that their flexibility is extremely reduced, thereby having relevant implications regarding their functional properties. IgYs from some species present ellipticity values (by circular dichroism) appreciably differing from those observed in IgG (12), indicating differences in secondary structure between both types of antibody.

IgYs have some physicochemical and functional characteristics differentiating them from mammalian IgG (13). Additio-

nally, IgY (differently to IgG) do not interact with rheumatoid factors (14), in such a way that the probability of returning false positives in immunochemical assays becomes reduced, do not bind to staphylococcal protein A (15), to protein G or to the human complement system (16). Such differences, added to the existing evolutionary distance, leading to the greater immunogenicity of mammalian proteins injected into birds (7, 17), and the considerable amounts of IgY produced by chicken during their egg-laying period (150-225 mg/yolk) provide important advantages when compared to mammalian IgG, which is why their use has markedly increased.

A lectin isolated from *Salvia bogotensis* (SBoL) has been recently described as part of our group's (GRIP) study of lectins in Colombian Lamiaceae which is able to specifically recognise the Tn antigen (GalNAc- α -Ser/Thr) (18); when this antigen is expressed on erythrocyte surface it causes the Tn syndrome, or polyagglutinability syndrome (19). The Tn antigen is frequently present on the external membrane of tumour cells and its abundance seems to be closely related to the aggressiveness with which cancerous tissue develops (20). However detection of the interaction SBoL-Tn antigen on cell surfaces requires a specific tool that recognizes SBoL; bearing in mind the advantages of IgYs, we have obtained and purified these immunoglobulins using SBoL as antigen (8) to make such tools available. The present work was aimed at molecularly characterising these IgYs and evaluating their application in immunochemical studies for detecting Tn antigen in tumour cells.

MATERIALS AND METHODS

General methods

The obtention and purification of anti-SBoL IgYs were described in (8) as well as the Dot-blot and protein quantification methods. Electrophoresis (PAGE-SDS) was done according to Laemmli's method (21).

Determination of IgYs molecular properties

Isoelectric point

The isoelectric-focusing method (IEF) as described by Bollag and Edelstein (22) was used in non-denaturing conditions. The pI value was established with isoelectric point protein standards ranging from 3 to 10 pH on 8% polyacrylamide gel (T₈). Before sowing the sample, the gel was pre-run at 400 V, 15 W for 20 minutes for establishing the pH gradient. 20 μ g of IgY dissolved in 2 μ l of ampholytes solution were then sown and run at 400 V, 15 W for 3 h. The gel was then placed in 10% trichloroacetic acid for 1 h for fixing and then in 1% trichloroacetic acid for 24 h or longer for washing the ampholytes from the gel. Coomassie blue solution was used for 15 minutes for staining.

Molecular weight

Molecular weight (MW) was determined by PAGE-SDS according to (21) in denaturing conditions without a reducing agent for determining antibody weight and with a reducing agent for separately establishing the weight of H and L chains. Known MW standards were used; electrophoretic mobility was plotted against the logarithm of MW.

Carbohydrate content

The method described by Dubois *et al.* (23) was used on a micro-scale. The assay was done in duplicate for two different amounts of IgY using a 96-well plate. Two glucose standards were used for the calibration curve (1 mg/ml and 3 mg/ml); IgY samples at known concentration were taken to 30 μ l desionised water. 30 μ l of phenol (5% in water) per well were then added. The plate was placed in triturated ice, left to cool for 15 min and 200 μ l of H₂SO₄ pre-chilled to -20 °C were added. It was left to stand for 15 min, incubated at 37°C for 12 h; absorbance was read at 490 nm.

Obtention of heterologous lectins

Lectin from Amaranthus hypocondriacus

A. hypocondriacus lectin was purified according to the method developed by Pérez (personal communication). The seeds were supplied by CINVESTAV (México) and ground up for obtaining the flour. Three extractions were carried out in a ratio of 1 g of flour / 10 ml 1% NaCl at 4 °C with constant shaking overnight. Fractioning with 30-75% ammonium sulphate was done with the pool of extracts; the protein pellet was recovered by centrifuging (15,000 rpm) for 20 min at 4 °C. The pellet was dissolved and dialysed 3 times against demineralised water. 10X PBS (1/10 of total volume) were added to the dialysed product and centrifuged (15,000 rpm) for 20 min; the supernatant was applied to an Agarose-Fetuin column (1x10 cm). The non-retained fraction was eluted with PBS and the retained fraction with a solution of 20 mM 1.3 diaminopropane, 10.52 pH. The retained fraction

was dialysed 3 times against 20 mM ammonium bicarbonate and lyophilised. Lectin purity was evaluated by SDS-PAGE (21). Activity was evaluated by agglutination assays for pure protein with T-erythrocytes.

Obtention of other lectins

The lectin from *Galactia lindenii* was obtained by following the methodology described by Almanza *et al.* (24); *Dioclea lehmanni* lectins were obtained according to Pérez *et al.* (25) for type I lectin and according to Pérez (26) for type II lectin. Type I *Dioclea grandiflora* lectins were obtained according to Moreira *et al.* (27) and type II ones according to Melgarejo *et al.* (28). *Erythrina edulis* lectin was isolated according to (29).

Obtention of homologous lectins

The *S. bogotensis* lectin (SBoL) was obtained following the procedure described by Vega and Pérez (18); *Salvia hispanica* and *Lepechinia bullata* lectins were also purified by this method.

Activity assays for lectins

Agglutination assays were carried out for pure lectins according to (29) using microtitre plates and (depending on the case) 2% erythrocytes from blood group H (O) or erythrocytes enzymatically degraded with sialidase according to (30) or 2% rabbit erythrocytes. Pure proteins were quantified by bicinchoninic acid method (31).

Interaction between the antibody and homologous and heterologous lectins

Interactions were evaluated by ELLSA (enzyme-linked lectin sorbent assay) method leading to determining the magnitude of interactions between IgYs and homologous Lamiaceae (*S. bogotensis*, *S. hispanica* and *L. bullata*) or heterologous lectins (*G. lindenii*, *A. hypocondriacus*, *D. lehmanni*, *D. grandiflora*, *E. edulis*) which were used for detecting eventual non-specific interactions. The plates were sensitised with each of the lectins (30 µg/ml); the methodology described in (18) was followed using IgY stock solutions (0.5 mg/ml), making serial dilutions to 100 µl / well final volume. Interactions were detected with a secondary Ab (chicken anti-IgY) produced in goat, coupled to peroxidase (Sigma) using ABTS as substrate; Abs were measured at 415 nm.

Detection of Tn antigen in MCF-7 and HeLa cell-lines

Labelling purified IgY with biotin

The methodology described in (32) was followed. Labelling was done in PBS pH 7.5 in a 1:2 antibody:biotin ratio (NHS-LC-biotin), adding the biotin to the antibody and leaving the reaction for 24 h. Residual biotin was eliminated by dialysis, making three changes with PBS. Labelled antibody was quantified (31) and direct Dot-blot was carried out for ascertaining its labelling (33). Biotinylated antibody activity was evaluated by ELLSA assays with SBoL.

Labelling purified IgY with peroxidase

The methodology described by Hermanson (34) was followed. Peroxidase (20.3

mg) was oxidised with sodium metaperiodate (88 mM) in PBS, pH 7.5, for 1 h, at 100 µl oxidising agent: 1 ml protein ratio. Oxidised peroxidase was dialysed against 0.2 M NH₄HCO₃, pH 9.6. Antibodies were labelled in 0.2 M NH₄HCO₃, pH 9.6, at 1:20 antibody:peroxidase ratio, adding oxidised peroxidase to the antibody and leaving the reaction for 2 h at room temperature. The conjugate was dialysed against PBS and labelled antibody was quantified (31); its activity was evaluated by ELLSA assays with SBoL.

Cell culture

MCF-7 (human breast adenocarcinoma) and HeLa (cervical cancer) cell-lines were cultured for being used as positive targets for detecting Tn antigen. The cells were cultured at 37 °C in a humid atmosphere and 5% CO₂; the culture medium used was DMEN, supplemented with 10% bovine foetal serum (SFB) and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin and 25 ng/ml amphotericin). Assays were carried out with cells having a maximum of 10 passages.

Detection of Tn antigen in MCF-7 and HeLa cell-lines with the lectin and labelled IgYs

CELISA detection of Tn antigen in cell-lines

Tn antigen was detected by CELISA using the technique described by Walker *et al.* (35). Cultured cells (10,000/well) were fixed with 4% paraformaldehyde, incubated with the lectin and then with dilutions of labelled antibodies. Controls for detecting eventual non-specific reactions were carried out as follows: cells +

revealer, cells + secondaryAb + revealer, cells + lectin + secondaryAb + revealer, cells + primary Ab + secondary Ab + revealer.

Immunocytochemical detection of Tn antigen in cell-lines

The assays were carried out according to the methodology followed in the Colombian NIH Neurosciences laboratory. Tn antigen was detected with SBoL (50 µg/ml) and labelled antibodies. The methodology described in (33) was followed.

RESULTS AND DISCUSSION

Determination of some molecular properties of antibodies

The molecular weight of the H and L chains as well as that of non-reduced antibody (purified by thiophilic chromatography) was determined by PAGE-SDS. A 69.4-71.3 kDa molecular weight was found for the antibody's heavy chain and 26.7-25.8 kDa for its light chain (Figure 1a). The range of MW variation was consistent with the technique's resolution and the lesser staining with Coomassie observed for the light chains is usual in these Igs (36). Non-reduced antibody had 163.9-167.1 kDa molecular weight (Figure 1b). These values were similar to those found by Leslie and Clem (5) by equilibrium sedimentation and gel filtration for intact IgY H and L chain molecular weights (170, 67.5 and 22 kDa, respectively) and those reported by mass spectrometry (37) (167.250, 65.105 and 18.660 Da). The absence of other bands suggest the purity of the IgY obtained. The MW observed for both intact protein and its constituent chains were greater

than respective MW in IgG; the small differences observed between the samples analysed here were probably due to microheterogeneity during glycosylation.

77.4 µg or 381.6 µg of protein were taken for quantifying carbohydrates giving

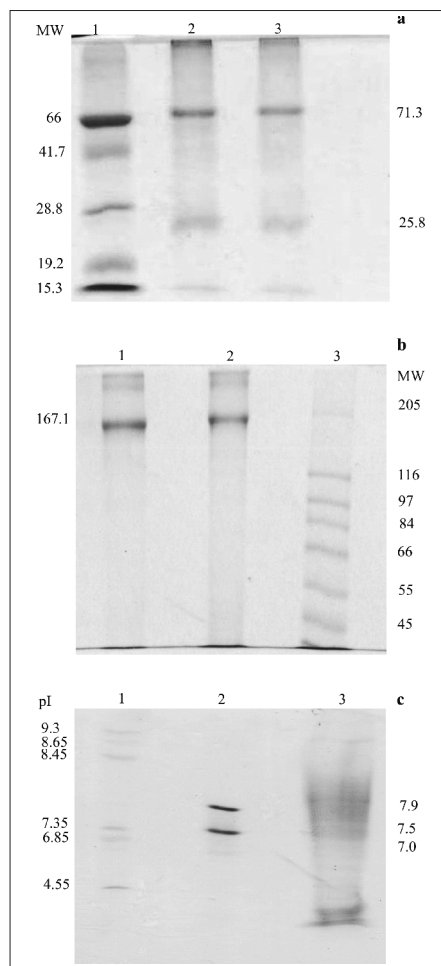


Figure 1. a. PAGE-SDS of reduced IgY. Lane 1: MW standards (pre-stained); lanes 2 and 3: IgY heavy and light chains. b. PAGE-SDS of unreduced IgY Lane 3: MW standards (pre-stained); lanes 1 and 2: IgY samples. c. Determination of IgY pI. Lane 1: pI standards; lane 2: myoglobin; lane 3: IgY.

an average of 3.11 μg and 15.1 μg of carbohydrate, corresponding to 4.0% and 3.9% of neutral sugars, respectively. The IgY carbohydrate content found in this work was close to that described using very precise methods, such as MS, where structural studies of oligosaccharides present in N-glycosylation sites (38) have given 8.560 Da MW per IgY molecule, corresponding to 4.7-5% carbohydrates per 180-170 kDa molecule. Hadge *et al.* (39), have reported values ranging from 4.6-6.4% for total hexoses, hexosamines and sialic acid. The lowest value (2.2%) reported by Leslie and Clem (5) was probably due to the isolation and quantification methodology used by these authors. Our data corroborated the differences with IgG where glycosylation was 2.3%.

IgYs presented isoelectric point values ranking from 6.5-7.9 pH (Figure 1c); given the polyclonal nature of these Ab and their microheterogeneity, these pI values agree very well with those found by Cheung *et al.* (40) and Gee *et al.* (41). Cheung *et al.*, (40) have determined 6.0-8.5 pI values for IgY; Gee *et al.* (41) have reported pI ranging from 6.5-7.5, being practically equal to those found by ourselves. The fact that there was a relatively broad range of pI was probably due to the antibodies being polyclonal; the heterogeneity produced by somatic diversification was manifest and it has been established that this occurs in the L chain gene (42).

Antibody interactions with homologous and heterologous lectins ELLSA assay

Erythroagglutination assays were positive in all cases, demonstrating that the lec-

tins used in this and other assays conserved their activity. Anti-SBoL IgY interaction with the panel of lectins from the Lamiaceae family (Figure 2a) presented high detection levels with the *S. bogotensis* lectin (as expected) and this was corroborated with *S. hispánica* lectin which belongs to the same genera and is specific for Tn antigen. 1 μg of IgY was sufficient for clearly detecting observable absor-

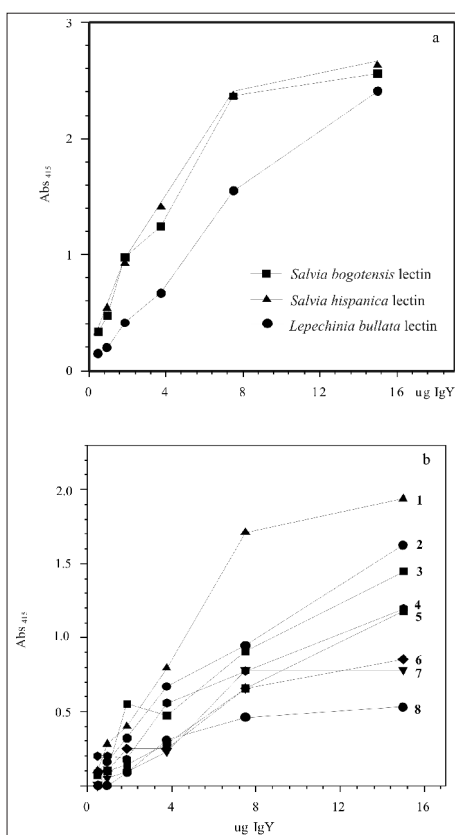


Figure 2. a. Antibody interaction with homologous Lamiaceae lectins evaluated by ELLSA assay. b. Antibody interaction with heterologous lectins evaluated by using an ELLSA assay. Lectins: *A. hypochondriacus* (1), *G. lindenii* type-II (2), *G. lindenii* type-I (3), *E. edulis* (4), *D. grandiflora* type-I (5), *D. lehmanni* type-I (6), *D. lehmanni* type-II (7), *D. grandiflora* type-II (8).

bance ($Abs_{415} \sim 0.500$) with these two lectins. These results indicated that there was high homology in these two lectins' structure on being almost identically recognised by the antibody. A somewhat lesser interaction was observed with *L. bullata* lectin (from another Lamiaceae genera) which is equally specific for Tn antigen, showing this lectin's lesser structural homology with *S. bogotensis* lectin, even though this clearly represented cross reactivity. This could be considered as being an advantage as these lectins can be used with anti-SBoL IgY for detecting Tn antigen in immunocytochemical and other tests.

On the other hand, the results revealed considerably lesser interaction with lectins from other families, particularly with those isolated from Leguminosae (Figure 2b); the weakest interactions were presented with *D. lehmanni* and *D. grandiflora* type II lectins, requiring 8 and 17 μg IgY (respectively) for obtaining clearly discernible absorbencies. An intermediate interaction (Figure 2b) was observed with *A. hypochondriacus* lectin regarding the other two families; this lectin is specific for antigen T (Gal β 1,3 GalNAc- α -Ser/Thr) and belongs to the Amaranthaceae family. Detection of the interaction observed with heterologous lectins consistently required higher amounts of IgY; the interaction was probably due to recognition of glycosidic structures present in these glycoproteins sharing the heptasaccharide distinctive of vegetal glycoproteins, which was also found in some Lamiaceae lectins (*S. bogotensis* (18), *Salvia sclarea* (43) and *Glechoma hederacea* (44)). These results indicate that it is convenient to use small amounts of IgY for specifically detecting (via SBoL) Tn

antigen, this being experimentally advantageous.

Biotin and peroxidase labelling of purified IgY and evaluation of its activity

Anti-SBoL antibodies were labelled with biotin or peroxidase for detecting Tn antigen for using them in cell interaction assays. Labelling was checked by evaluating biotinylated IgY by Dot-blot, observing a positive signal with 0.05 μg of protein (Figure 3a). IgY-labelled functionality assays (992 μg), carried out by

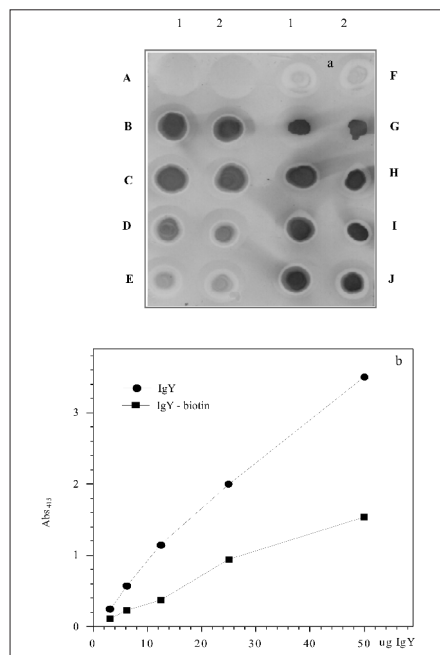


Figure 3. a. Dot-blot of IgY. Dots A1, A2: blank; B1, B2: 500 μg of biotin-labelled IgY; C1, C2: 50 μg of biotin-labelled IgY; D1, D2: 5 μg of biotin-labelled IgY; E1, E2: 0.5 μg of biotin-labelled IgY; F1, F2: 0.05 μg of biotin-labelled IgY; G1, G2: 1300 μg of biotin-labelled IgY; H1, H2: 620 μg of biotin-labelled IgY; I1, I2: 310 μg of biotin-labelled IgY; J1, J2: 150 μg of biotin-labelled IgY. **b.** IgY: lectin and biotin-labelled IgY: lectin interactions assayed by ELLSA.

ELLSA, showed that, on interacting with SBoL, around 17 μg of IgY-biotin presented 0.5 A_{415} (Figure 3b). On the other hand, 3 and 0.7 μg of protein were required with IgY-peroxidase for obtaining 0.275 and 0.105 A_{415} , respectively (data not shown). These results showed that functionality of IgY was preserved after labelling.

CELISA detection of Tn antigen in MCF-7 and HeLa cell-lines

These lines were chosen as experimental model, given that Tn antigen has been previously detected in them with biotinylated SBoL and antibodies produced in rabbit against the lectin (33). In the present work assays were done (via SBoL recognition by anti-SBoL IgYs) for detecting Tn antigen in cells and evaluating possible IgY crossed reactivity with antigenic cell surface determinants. All controls gave negative results, showing the absence of non-specific interactions between IgYs and cells.

Using 1 μg of biotin- or peroxidase-labelled IgY led to obtaining similar detection values for the MCF-7 line ($A_{415} \sim 0.800$) from Tn antigen (Figure 4a), lower absorbance values being observed with the same amount of unlabelled IgY. Levels of detecting Tn antigen were also high with the HeLa line and were clearly discernible with 2 μg of labelled IgYs (Figure 4b). These results indicate that Tn detection can be achieved with high sensitivity with native lectin avoiding potential artifacts derived from labelled SBoL. It was observed with both lines that the

magnitude of the interaction was similar when using IgY-biotin or IgY-peroxidase as detection system, indicating that they did not differ appreciably regarding the amount of Tn antigen detected on their surface. When greater amounts of labelled IgY (1-50 μg) were used, it was observed with MCF-7 that more than 30 μg of biotinylated IgY seemed to saturate the cell system (data not shown); this agreed with that described by (33) when biotinyl-

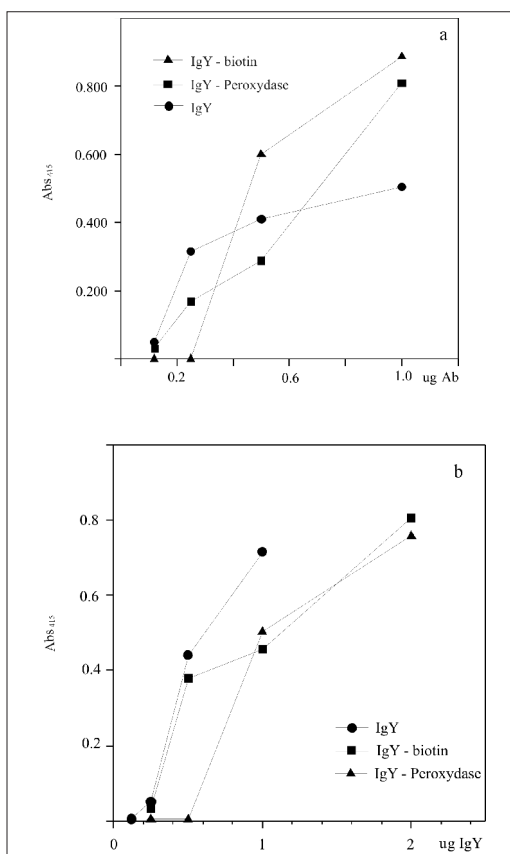


Figure 4. a. Detection of Tn antigen in the MCF-7 cell-line by CELISA assay using IgY, biotin-labelled IgY and peroxidase-labelled IgY. b. Detection of Tn antigen in the HeLa cell-line by CELISA assay using IgY, biotin-labelled IgY and peroxidase-labelled IgY.

lated SBoL was used for detecting Tn antigen.

The results of detecting Tn antigen obtained with biotinylated IgYs in ELLSA seem to indicate a reduction in antibody activity compared to those obtained by CELISA. Such discrepancy can very probably be explained by the inherent differences in the two assays as Tn antigen density in the sub-maxillary bovine mucine isolate used in the ELLSA assay is not the same as in tumour cells.

In any case, the CELISA assay results showed that anti-SBoL IgY could be used for detecting with high sensitivity Tn antigen in tumoral lines, as SBoL recognition by antibodies was clear and specific.

Immunocytochemical detection of Tn antigen in cell-lines

Cells having abnormal nuclei (typical of pre-established lines) were observed in the cell-lines; in most MCF-7 cells labelling in the perinuclear region was observed with SBoL and the antibody (rough endoplasmatic reticle), where there was glycoprotein secretion and labelling intensity was almost homogeneous in the cytoplasm (Figures 5a, 5b). Figures 5c, 5d presents the controls where non-specific labelling was not observed. Similar results were obtained with HeLa cells (data not shown). The same labelling pattern was seen with biotinylated SBoL in other work carried out in the group with both these lines (33) and only the centrosomal

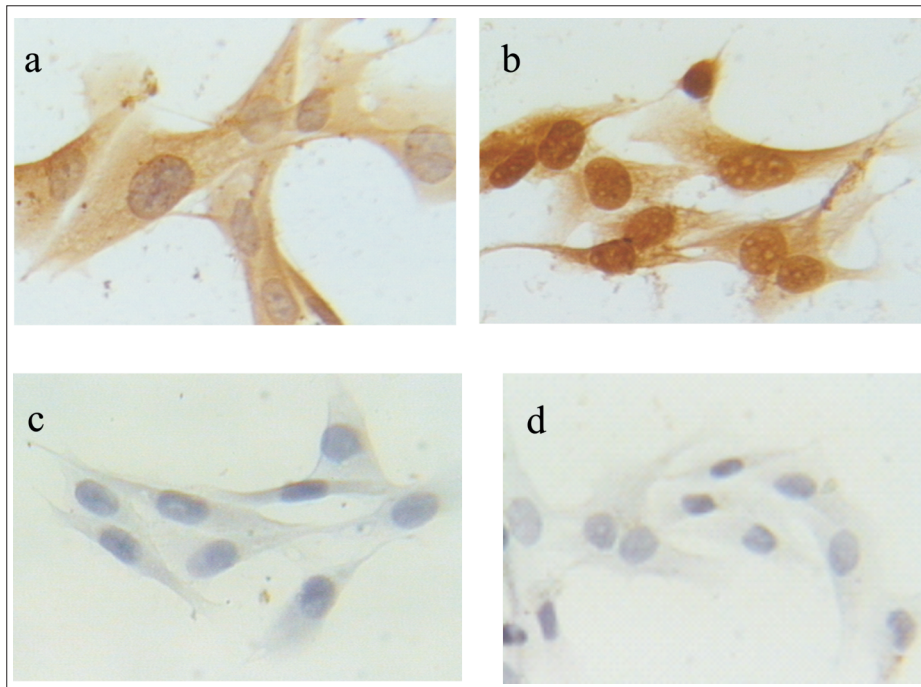


Figure 5. a. b. Detection of Tn antigen in MCF-7 cells by immunocytochemistry using a) biotin-labelled IgY and b) peroxidase-labelled IgY. c. d. Immunocytochemistry assay controls without antibody.

complex was labelled with B₄ isolectin from *Vicia villosa* (specific for Tn antigen); labelling in the centrosomal complex was also observed in the perinuclear area and in the cytoplasm with AbMo 83D4 (anti-Tn). On the other hand, no labelling was detected on periplasmatic membrane.

Our results showed that the lectin recognised glycoproteins from cytoplasm in which Tn antigen was found to be present; it can be speculated that they are mucin-like proteins but the exact nature of these proteins remains to be elucidated.

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